The application was originally made under the Patent Cooperation Treaty with the Japanese Patent Office acting as the receiving office on (86) 27 June 1981, being given an application number PCT/JP81/00148. The application was searched by the Japanese Patent Office acting as the International Search Authority (ISA), and published by the International Bureau on (87) 21 Jan 1982 under serial number WO82/00096 in the Japanese language. The text of the application is contained in the publication made by the International Bureau as above identified. The accompanying text being being an English translation thereof

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# (54) Thrombosis-prophylatic and curing agent

(57) A thrombosis-preventing and curing agent containing at least one member selected from among (all-Z)-4,7,10,13,16,19-docosahexaenoic acid, pharmaceutically acceptable salt, ester and amide thereof as effective ingredient. This agent is absorbed through the intestine so well that it can be used internally, is stable in blood and shows excellent effect of preventing blood platelets from agglutinizing.



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FIG_1

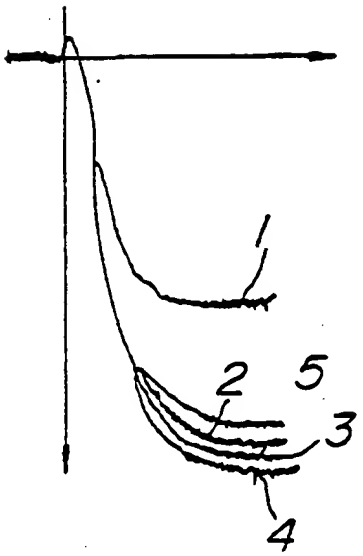


FIG_2

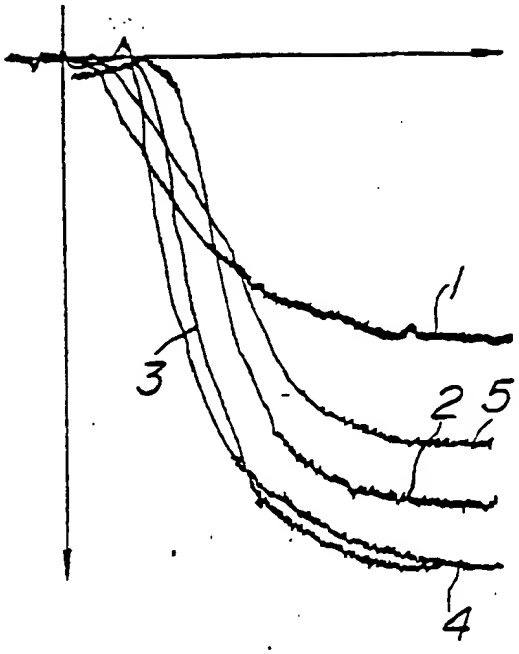


FIG_3

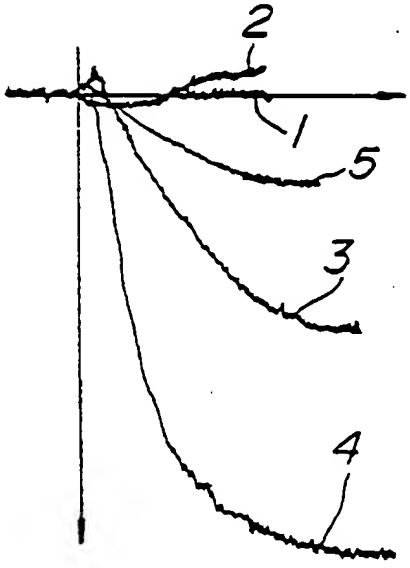


FIG_4

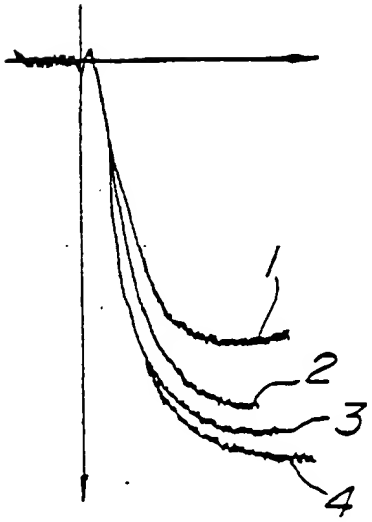

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FIG.5

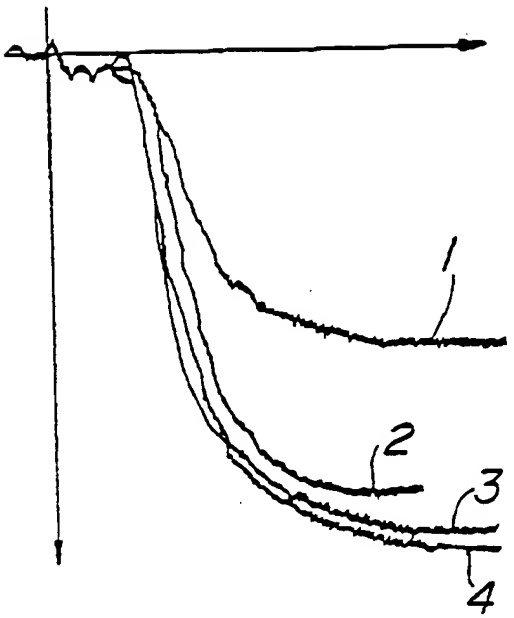


FIG.6

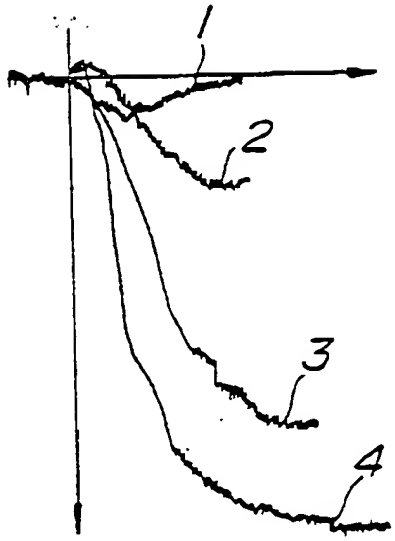


FIG.7

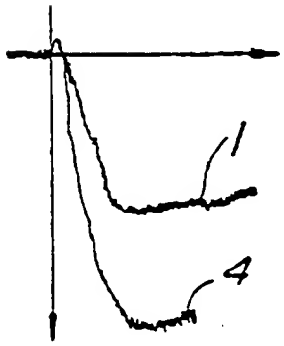


FIG.8

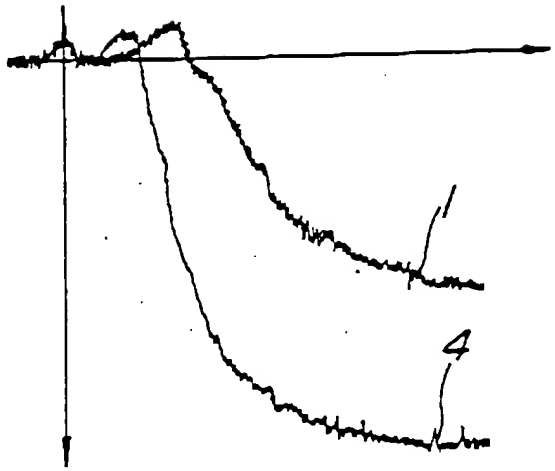

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FIG_9

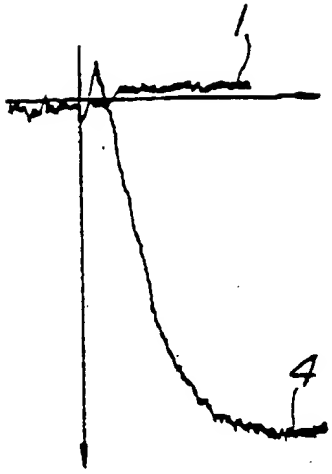



FIG_10

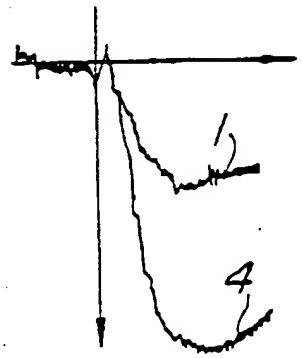



FIG_11

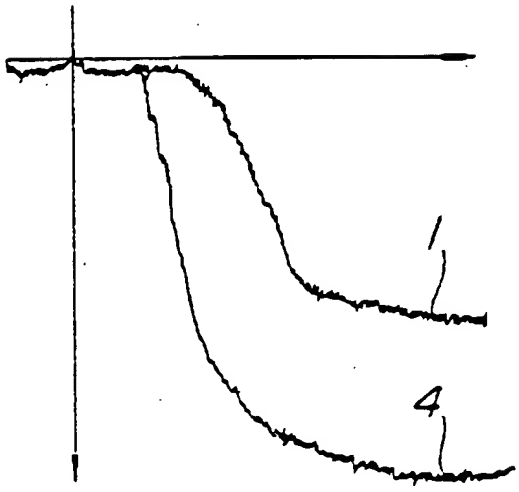
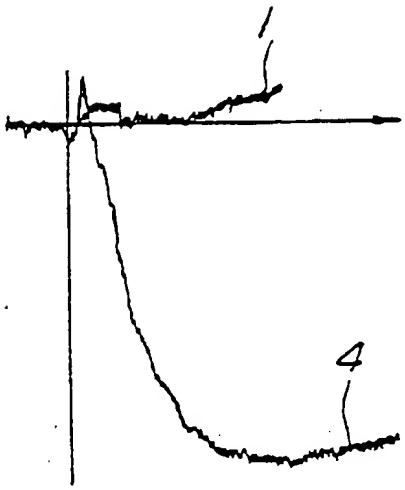



FIG_12



SPECIFICATION

# Formulations for prophylaxis or treatment of thrombosis

## TECHNICAL FIELD

The present invention relates to formulations for prophylaxis or treatment of thrombosis.

## BACKGROUND ART

Thrombosis means a pathologic phenomenon wherein platelet is aggregated and blood is coagulated in heart or blood vessel of organisms to form coagulum or thrombus and the formation of thrombus brings about constriction and obstruction of blood vessel and causes change to isochemic and infarct in main organs, such as heart, brain, pulms and the like and brings about the functional hindrance of these organs and causes clinical important disease. The cause of the formation of the above described thrombus has not been heretofore clarified but the change of the blood vessel wall condition, the change of the blood flow and the change of the blood ingredients are considered to be fundamentaly the main causes and recently the aggregation factor, the fibrinolytic factor, adhesion and aggregation factor of platelet, such as prostagradine etc. and reticuloendothelial system have been noticed to have relation to the above described thrombus or the blood coagulation in blood vessels. Thus, the thrombosis is caused owing to abnormally close and complicated entanglement of the above described various causes but in any case, the blood coagulation is observed in the blood vessels, so that as the formulations for the prophylaxis or treatment, use has been made of chemicals, that is anticoagulants which act on the reaction system in the course of coagulation of blood and inhibit the blood coagulating mechanism and remove the factors concerning thereto and reduce the coagulating property of the blood. As the representative ones, sodium citrate, heparin, cumarine derivatives, indandione derivatives etc. have been known. These anticoagulants, considering from the functional mechanism, have no activity for dissolving the already formed thrombus and therefore these substances aim to improve the progress of the blood coagulation and are only effective for prophylaxis of generation of the thrombosis or prevention of regeneration of the thrombosis. Recently, enzyme formulations, such as urokinase, streptokinase etc. have been developed as substances which positively dissolve the thrombus and improve the blood flow, that is thrombus dissolving agents, and particularly urokinase having neither antigenicity nor pyrogenic property has been broadly used clinically. It has been pointed out that the platelet-hyperergasia has a close relation to the formation of the above described thrombus and for the prophylaxis or treatment of the platelet-hyperergasia, an inhibitor for thrombus function, that is antiplatelet, for example chemicals which inhibit the formation or aggregation of LASS (Labile aggregation stimulating substance), such as a thrombus membrane stabilizer, adenylcyclase activator, phosphodiesterase inactivator, aspirin, non-steroid series antiphlogistic and the like, has been proposed, but the effectiveness of these substances has not been yet confirmed. The prophylaxis or treatment of the thrombo-embolic conditions has been disclosed in Japanese Patent Laid Open Application No. 154,533/79 and this relates to a formulation for prophylaxis or treatment of the thrombosis wherein the active component is eicosapentaenoic acid etc. However, it has been found that the formulations of the present invention wherein the active component is docosahexaenoic acid etc. have higher activity for the prophylaxis or treatment of thrombosis than these formulations in the above described prior art.

## DISCLOSURE OF INVENTION

The inventors have made diligent studies with respect to clarification of the functional mechanism of the above described well known various formulations for prophylaxis or treatment of thrombosis and the mechanisms of blood coagulation and the mechanism of fibrin dissolution, which become the premise of the above described functional mechanism and found that a certain highly unsaturated fatty acid or the derivatives thereof which are different from the already known various chemicals, have the function for inhibiting the blood coagulation, that is the platelet aggregation induced by arachidonic acid, adenosine disphosphate (ADP) or collagen, and the function for dissolving the platelet aggregate, and that the functional effect is more than twice higher than eicosapentenoic acid and the active components of the present invention are effective for the prophylaxis or treatment of thrombosis. The present invention has been accomplished based on this novel discovery.

Namely, the present invention consists in the formulations for prophylaxis or treatment of thrombosis characterized in that at least one of (all–Z)–4, 7, 10, 13, 16, 19-docosahexaenoic acid and the pharmaceutically acceptable salts, esters and amides of this acid is contained as the active component.

Since the formulations for prophylaxis or treatment of thrombosis according to the present invention use the above described specific higher unsaturated acid and the derivatives thereof as the active component, they show excellent effect in prophylaxis or treatment of various thrombosises, such as deep venous thrombosis, limb artery thrombosis, embolism, pulms embolism, venous thrombosis of retina, coronary occlusion, cerebral thrombosis and the like, and myocardial in-

farction, acute cardiac insufficiency, apoplexy and the like, which are induced from the above described thrombosises. In particular the above described active components are high in the absorbability through intestine vessels and can be orally administered and are stable in blood and therefore, the formulations can maintain the activity for a long time and be administered in a fairly large quantity or for a long time but the growing stout tendency due to the excessive calory based on such an administration does not occur.

(all-Z)-4, 7, 10, 13, 16, 19-docosahexaenoic acid used as one of the active components in the present invention is contained in marine animal oils and can be isolated from marine animals through usual methods, for example molecular distillation method, countercurrent distribution method, chromatography and the like and a part of said acid is commercially available as a standard reagent. The higher unsaturated acid obtained from marine animals is not necessary to be the isolated purified substance for the practical use and may be a crude product containing a small amount of the other higher unsaturated acids etc. Furthermore, the above described compound may be ones produced by organic synthetic production from a proper starting material. In the present invention, the pharmacentrically acceptable salts, esters and amides of the above described compound can be used as the active component as well as the above described compound. As the pharmacentically acceptable salts and esters, the representatives are alkali metal, alkaline earth metal and other metal salts, such as sodium salt, potassium salt aluminum salts etc., ammonium salt, amine salts, such as norpholine, piperadine, trimethylamine, diethylamine etc., lower alcohol esters, such as methyl ester, ethyl ester etc.

In the formulations for prophylaxis or treatment of thrombosis of the present invention, the active component may be administered alone but is usually administered in the form of formulations together with carriers. As the carriers, diluents or vehicles which are usually used for preparation of formulates depending upon the using form, such as fillers, extenders, binders, humidifiers, disintegrators, surfactants, lubricants and the like are examplified. The formulations may be administered in a variety of forms, for example tablets, pills, powders, liquids, suspensions, emulsions, granules, capsules, suppositories, injections (solutions, suspensions etc.) and the like. For formation of tablets, as carriers, use may be made of vehicles, such as lactose, cane sugar, sodium chloride, glucose, urea, starch, calcium carbonate, kaolin, crystalline cellulose, silicic acid etc., binders, such as water, ethanol, propanol, syrup, glucose, glycol, glycerin, starch solution, gelatin solution, carboxylmethyl cellulose, shellac, methyl cellulose, potassium phosphate, polyvinyl pyrrolidone etc., disintegrators, such as starch, sodium alginate, agar powder, laminaria powder, sodium hydrogencarbonate, calcium carbonate, twin, sodium laurylsulfate, monoglyceride stearate, lactose and the like, disintegrate inhibitors, such as cane sugar, stearin, cacao butter hydrogenated oils etc., adsorption accelerators, such as quaternary ammonium salts, sodium laurylsulfate etc., humidifiers, such as glycerin, starch etc., adsorbents, such as starch, lactose, kaolin, bentnite, colloidal silicic acid etc., lubricants, such as purified talc, stearates, boric acid powder, solid polyethylene glycol etc. For formation of pills, as carriers, use may be made of vehicles, such as glucose, lactose, starch, cacao fat, hardened vegetable oils, kaolin, talc etc., binders, such as Arabian rubber powder, tragacanth powder, gelatin, ethanol etc., disintegrators, such as laminaria, agar etc. Tablets may be used by applying a usual coating, if necessary, for example in sugar-coated tablets, gelatin-coated tablets, intestine soluble coated tablets, film-coated tablets or double layer tablets or multi-layer tablets. For formation of suppositories, as carriers, use may be made of polyethylene glycol, cacao fat, higher alcohols, esters of higher alcohols, gelatin, semi-synthesized glyceride etc. When injections are prepared, it is preferable that the solutions or suspensions are sterilized and are made to be isotonic to blood, and for preparation of solution, emulsion and suspension formulations, as diluents, use may be made of water, ethyl alcohol, propylene glycol, ethoxyisostearyl alcohol, polyoxyisostearyl alcohol, polyoxyethylene sorbit, sorbitan esters etc. In this case, an enough amount of salt, glucose or glycerin to prepare the isotonic solutions may be contained in the formulations. For preparation of paste, cream or gel formulations, as diluents, use may be made of white vaseline, paraffin, glycerin, cellulose derivatives, polyethylene glycol, silicone, bentnite etc. In addition, in the formulations for prophylaxis or treatment of thrombosis of the present invention, antioxidants, such as hydroxytoluene butyrate, propyl gallate, quinone, α-tocopherol etc., usual dissolution aids, buffer agents, agent causing no pain, preservatives, coloring agents, perfumes, flavorings, edulcorants and other pharmacentical agents may be contained. Some antioxidants promote the effect for preventing thrombus.

A quantity of the active component contained in the formulations is not particularly limited but can be properly selected in a broad range and is generally at least 1% by weight of the total formulation and for example, the tablets contain about 0.2-1 g of the active component per one tablet, which is calculated as the free acid.

The formulations for prophylaxis or treatment of thrombosis according to the present

invention may be administered in the proper manner which is not particularly limited, depending upon the form of the formulations. For example, the tablets, pills, solutions, suspensions, emulsions, granules and capsules may be orally administered, the injections may be administered intravenously alone or together with usual aids, such as glucose, amino acids etc. and if necessary, administered alone intramuscularly, subcutaneously or intraperitoneally, and the suppositories may be intrarectumly and in the case of women, intraveginaly. The quantity of the formulations is properly selected depending upon the administering manner, the condition of patients and the like and in general, the quantity of the active component calculated as the free acid is about 10–50 mg/kg.day, preferably about 20–40 mg/kg.dey and this is usually divided in 3–4 times in day and administered.

In addition, the active component of the present invention may be given in the necessary amount to the patients in the form of glyceride as margarin, butter, a cooking oil or fat and therefore, the present invention provides the formulations for prophylaxis or treatment of thrombosis in a food form containing such a fat and oil.

Thus, the present invention can provide the formulations for prophylaxis or treatment of thrombosis which have never been heretofore found.

## *BRIEF EXPLANATION OF THE DRAWINGS*

*Figure 1–Fig. 12* are graphs showing the variation of turbidity of the blood specimens used in the experiments for showing the inhibiting function of the compounds according to the present invention with respect to the aggregation of platelet induced by ADP, collagen and arachidonic acid respectively and in each drawing,

(1) shows the specimen compound concentration of 500 μg/ml,

(2) shows the specimen compound concentration of 250 μg/ml,

(3) shows the specimen compound concentration of 125 μg/ml,

(4) shows the control solution and

(5) shows EPA concentration of 500 μg.ml.

## *PREFERRED EMBODIMENT FOR CARRYING OUT INVENTION*

The present invention will be explained with reference to experiments:

*Experiment 1*

*Reagent*

As (all–Z)–4, 7, 10, 13, 16, 19-docosahexanenoic acid of the active component of the present invention, a product made by KOWA JUNYAKU KOGYO CO. was used in an ethanol solution of each of 500 μg/ml, 250 μg/ml and 125 μg/ml. As a comparative specimen, an ethanol solution of 500 μg/ml of (all–Z)–5, 8, 11, 14, 17-eicosahexaenoic acid (referred as EPA) was used.

As collagen of a pro-aggregator of platelet, a physiologic salt solution of collagen made by Hormon Chemie Co. West Germany was used and as ADP and arachidonic acid, these compounds made by Sigma Co. were used in a physiologic salt solution and an ethanol solution respectively.

*Preparation of platelet rich plasma*

3.8% sodium citrate of a volume of 0.1 time as much as blood was fed through a catheter inserted into carotid artery of Japanese white rabbit under non-narcosis as an antiaggregator and the blood was taken out. Said blood was subjected to a centrifugal separator at 1,100 rpm for 10 minutes to separate a supernatant liquid and the precipitated residue was again subjected to the same separator as described above at 3,000 rpm for 15 minutes to separate a supernatant liquid, which is referred to as PPP (platelet poor plasma). A number of the platelet in the plasma obtained in the centrifugal separation under the above described 1,100 rpm was measured by Coulter counter–ZB–1 and this was diluted with the above described PPP so that the number of platelet becomes $5 \times 10^8$/ml to prepare PRP (platelet rich plasma).

*Platelet aggregation test*

Following to nephelometry method by Born et al described in Nature *194*, 927–929 (1962), the platelet aggregation test was carried out by means of Aggregometer model PAT—6M type made by NIKOKIZAI Co. as follows.

200 μl of PRP was charged into a cuvette in the above described aggregometer and 1 μl of ethanol solution of the compound to be tested and 1 μl of ethanol which is a control, were added thereto respectively and the mixtures were preincubated at 37°C for 1 or 5 minutes respectively, and 20 μl of ADP solution or 20 μl of collagen solution or 1 μl of arachidonic acid solution prepared into the given final concentration (ADP: 7.5 μM, collagen: 20 μg/ml and arachidonic acid: 50 μg/ml) with physiologic salt solution or ethanol were added thereto respectively to cause the platelet aggregation. The variation of the turbidity (variation of absorbance) of PRP was continuously recorded by means of the above described aggregometer to determine the activity for inhibiting the platelet aggregation of the compound to be tested in each concentration.

*Results*

The results are shown in Fig. 1–Fig. 6. Fig. 1–Fig. 3 show the activity for inhibiting the platelet aggregation induced by ADP (Fig. 1), collagen (Fig. 2) and arachidonic acid (Fig. 3)

*Preparation Example 1*

| | |
|---|---|
| (all–Z)–4, 7, 10, 13, 16, 19-docosahexaenoic acid | 140 mg |
| Starch | 31.4 mg |
| Lactose | 125 mg |
| Polyvinyl pyrrolidone | 1.8 mg |
| Magnesium stearate | 1.8 mg |
| Total | 300 mg |

CLAIMS

1. A formulation for prophylaxis or treatment of thrombosis comprising at least one of (all–Z)–4, 7, 10, 13, 16, 19-docosahexaenoic acid and pharmacentically acceptable salts, esters and amides of this acid as an active component.

2. A formulation as claimed in claim 1, wherein the active component is administered in a quantity of 10–50 mg/kg per day in weight as calculated as the free acid.

3. A formulation as claimed in claim 1, wherein the active component is contained in the form of glyceride as butter, margarine or cooling oil or fat.

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respectively when the preincubation time is 5 minutes, and Fig. 4–Fig. 6 show the similar activity for inhibiting the platelet aggregation when the preincubation time is 1 minute. In each drawing, the abscissa shows the time and the ordinate shows the variation of the absorbance. In each drawing, the curve 1 is the case of the compound concentration of 500 μg/ml, the curve 2 is the case of the compound concentration of 250 μg/ml and the curve 3 is the case of the compound concentration of 125 μg/ml, the curve 4 is the control (no addition of the test compound) and the curve 5 is the case of 500 μg/ml of EPA for comparison.

From Fig. 1–Fig. 6, it is apparent that the active compound of the present invention has the satisfactory inhibiting activity against the platelet aggregation induced by ADP, collagen or arachidonic acid even though the preferable concentration is somewhat different. From Fig. 1–Fig. 3 it can be seen that the active compound of the present invention has the inhibiting activity of about twice as much as EPA and the compound is effective for prophylaxis or treatment of thrombosis.

*Experiment 2*

The inhibiting activity of sodium salt of (all-Z)-4, 7, 10, 13, 16, 19-docosahexaenoic acid was examined by using 500 μg/ml of aqueous solution of this salt in physiologic salt solution in the same manner as described in Experiment 1 with respect to the platelet aggregation induced by ADP, collagen and arachidonic acid.

The results when the preincubation time is 5 minutes are substantially equal to those in Fig. 1–Fig. 3 respectively and the results when the preincubation time is 1 minute are similar to those in Fig. 4–Fig. 6 respectively.

*Experiment 3*

The same test as in Experiment 1 was made with respect to ethyl ester of (all-Z)-4, 7, 10, 13, 16, 19-docosahexaenoic acid in the form of 500 μg/ml of ethanol solution.

The result of the inhibiting activity to the platelet aggregation induced by ADP is shown in Fig. 7, the result of the inhibiting activity to the platelet aggregation induced by collagen is shown in Fig. 8 and the result of the inhibiting activity to the platelet aggregation induced by arachidonic acid is shown in Fig. 9. In each drawing, the curve 1 shows the compound to be tested and the curve 4 shows the control.

From these drawings, it can be seen that the compound of the present invention in the form of the ethyl ester also has the excellent activity for inhibiting the platelet aggregation.

*Experiment 4*

The same test as in Experiment 1 was made with respect to amide of (all-Z)-4, 7, 10, 13, 16, 19-docasahexaenoic acid in the form of 500 μg/ml of ethanol solution.

The results are shown in Fig. 10–Fig. 12. Fig. 10–Fig. 12 correspond to the above described Fig. 6–Fig. 9 respectively and the curves 1 and 4 in each drawing have the same meanings as in Fig. 6–Fig. 9. In each drawing, the preincubation time was 5 minutes. From Fig. 10–Fig. 12, it can be seen that the active compound in the form of the acid amide has substantially the same excellent inhibiting activity to the platelet aggregation as in the free acid (Fig. 1–Fig. 3) and the ethyl ester (Fig. 6–Fig. 9) and therefore, this compound is effective for prophylaxis or treatment of thrombosis.

*Experiment 5*

This experiment was followed to the method of Hornstra (Brit. J. Haemotol, *19*, 321 (1970)). 50 mg/kg of pentobarbital was administered intraperitoneally into Wister rat (male 250–350 g) to apply anesthesia and the abdomen was opened and a circulating passage was formed in abdominal aorta with a polyethylene tube and a part of said tube was exposed to the outside of the body and the abdomen was closed. 24 hours after the above described operation, the rat was bound to a secured plate and a filter (20 μm) inner diameter: 13 mm) was provided in the circulating passage. The circulating passage of the outside of the rat was filled with the physiologic salt solution containing heparin (200–400 unit) and held in a thermostat kept at 36 ± 1°C. From the force part of the filter, 1 μ/ml of aqueous solution of ADP in physiologic salt solution was administered in about 10 seconds to cause the platelet aggregation. The pressure at the fore part and the back part of the filter was measured and the difference is counted, which was adopted as an indication of the aggregation degree.

After ADP was administered 3 times in an interval of 30 minutes, the above described pressure differences were counted to determine the aggregation degree of the control and succeedingly, Arabian rubber suspension of the same (all-Z)-4, 7, 10, 13, 16, 19-docosahexaenoic acid as used in Experiment 1 was orally administered as the test compound in a dose of the active component of 100 mg/kg. 1 hour after the oral administration, the pressure difference at the fore part and the back part of the filter was again measured to determine the aggregation degree and this was compared with the aggregation degree of the above-described control to determine the inhibiting ratio of the test compound with respect to the platelet aggregation induced by ADP. Thus, it has been found that the above described compound inhibits 20% of platelet aggregation in average based on the control.